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Surgical Techniques and Biologic Growth Factors-CIC3

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FOREWORD

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STATEMENT OF WORK

Objective One: The impact of airway stenting on wound healing (**i.e. Phase One**) will be tested. This includes histologic and immunohistochemical analysis.

Objective Two: The impact of airway stenting with and without the application of a fibroblast inhibitor in the setting of airway injury (**i.e. Phase Two**) will be tested. This includes histologic analysis.

Objective Three: The impact of airway stenting with and without the application of a fibroblast inhibitor in the setting of augmentative airway reconstruction (**i.e. Phase Three**) will be tested. This includes histologic, immunohistochemical and scanning electron microscopic (SEM) analysis.

Objective Four: The impact of airway stenting with and without the application of a fibroblast inhibitor in the setting of sleeve resection of the airway (**i.e. Phase Four**) will be tested.

Introduction

Subglottic and tracheal stenosis frequently requires reconstruction with augmentative procedures. Success in these procedures, can be thwarted by aggressive postoperative wound healing, manifested by prominent granulation tissue and fibrosis.¹ Granulation tissue in this setting requires removal to prevent airway obstruction, as well as restenosis. This removal, usually via laser treatments, can induce airway stenosis and ultimately results in surgical failure. The use of an antiproliferative agent (MTC) at the time of airway reconstruction has shown significant reduction in postoperative scarring and granulation tissue formation. The effect of this agent on airway wound healing has not been prospectively assessed in an animal model. A better understanding of airway wound healing physiology should result in more predictable surgical outcomes.

Augmentative procedures to expand subglottic and proximal tracheal stenosis have included the use of cartilage and bone. Cartilage remains viable in the reconstructed airway, making it the preferred material for reconstruction in the pediatric airway.³ These reconstructive procedures involve the use of stents to maintain airway size and stability during healing. However, stenting is associated with inflammation and granulation tissue formation. Current surgical techniques advocate shorter periods of stenting to avoid this problem. Despite this trend short term stenting is not always possible. Previous studies have demonstrated the adequacy of the porcine model in airway reconstruction.⁴ We have been evaluating the impact of expandable metallic stents (Palmaz, Johnson & Johnson Interventional Systems Co.) in the pig airway.⁵ These stents were found to have no impact on tracheal growth and produce a predictable amount of inflammation, that is reversed with stent removal. In a recent pilot study these stents allow for a defined amount of airway expansion, as well as eliminating the need for intensive postoperative monitoring.³⁴ This has allowed single stage airway reconstruction in an animal model, without the need for tracheotomy.

Our understanding of the impact of antiproliferative agents in wound healing is in its infancy. MTC is a naturally occurring antibiotic-antimetabolite that inhibits fibroblast proliferation.⁶ It is used extensively in ophthalmologic surgery to prevent excessive scarring associated with trabeculectomy.⁷

Additionally it is used to prevent recurrence of pterygium, a proliferation conjunctival epithelium associated with a inflammatory fibrovascular response.⁸ Recent reports have also demonstrated benefit in wound healing modulation with this substance in respiratory epithelium.^{9,10} A single topical application of this substance allows rapid epithelialization, while preventing fibrosis. The exact process necessary for successful tissue repair of upper airway reconstruction.

Evaluation of airway inflammation and wound healing is difficult. Qualitative assessments can be made histologically and with immunohistochemical staining for growth factors. We have modified a histologic scoring system to objectively quantify airway inflammation induced by airway stenting.¹¹ Our preliminary data shows that this scale is a valid assessment of this inflammation, with a stent in place and after its' removal. Another means of evaluating airway inflammation is at the subcellular level through the detection of growth factors.¹² These substances induce cell proliferation, differentiation and synthetic activity. Much has been written on the role of these substances in tissue repair, but little literature exists describing their activity in airway wound healing. Combining our scoring system as well as an assessment of the tissue repair processes at a subcellular level is needed to further understanding of airway wound healing.

Repair of upper airway stenosis is frequently complicated by unpredictable wound healing. This study will attempt to further elucidate the physiology of this process. Expandable metallic stents will be used to create an experimentally expanded airway lumen of a known size. Additionally, these stents produce inflammation, while maintaining a patent airway, so airway reconstruction can be performed without the need for adjunctive measures for airway control. These factors create a realistic animal model for evaluating airway wound healing. Antiproliferative agents are already used in other types of surgery involving epithelial healing potential, but the mechanism of action of these substances in the upper airway needs systematic evaluation in an animal model. This project will provide such an evaluation and further our understanding of the impact that antiproliferative agents can have in modulating airway tissue repair.

Summary of Research

PHASE 1: Wound Healing in the Airway with Stenting Alone.

Introduction:

The use of expandable metallic stents to alleviate tracheobronchial obstruction has been well documented in the recent literature. They have been used in three scenarios; palliative management of airway compromise secondary to tumor compression, expansion of stenosed bronchial anastomoses following lung transplant and in treating severe tracheobronchomalacia in children.¹³⁻¹⁸ The clinical application of expandable stents in the airway has bypassed our understanding of the effect stents has on the respiratory tissue. This is especially true for in pediatric patients. For this reason their use in children is controversial.¹⁹⁻²³ Many unanswered questions remain, such as the effect of stents in airway growth and the cause of airway granulation tissue associated with their use.^{21,24} Few studies have evaluated the effects of expandable stents on tracheal tissue inflammation and wound healing utilizing an animal model. This study's purpose is to evaluate the impact of short-term stenting on tracheal tissue.

Design and Subjects:

A prospective, randomized, controlled study was performed on 32 ten-week-old piglets (*Sus Scrofa*) weighing 20-25 pounds each. Animals were divided into 4 groups of 8, seven experimental and one control. We used this animal model based upon the similarities between the piglet and infant airway. The Institutional Review Board and Animal Care Committee approved the research protocol at Madigan Army Medical Center, Tacoma, Washington and the Department of the Army. All animals were pre-medicated with Torbugesic 0.3mg/kg, Ketamine 20mg/kg, Rompin 2mg/kg and Atropine .02mg/kg. Intraoperatively, they were anesthetized with a continuous infusion of Propafol at 4-8 mg/kg/hr. All subjects received Cefazolin 500mg IM perioperatively and Torbugesic .3mg/kg intramuscularly post-operatively for

analgesia. Subjects were maintained and cared for by the Department of Clinical Investigations animal husbandry unit, perioperatively.

Interventions:

Animals underwent endoscopic evaluation of their airway, from the epiglottis to the carina, using a 3.5 rigid bronchoscope and 0 degree Hopkins II telescope (Karl Storz Endoscopy-America Inc.). Airway measurements were obtained at the tracheal take-off of the right upper lobe bronchus and carina. All control animals underwent the same rigid bronchoscopy protocol to include instrumentation with the exception of stent placement. The animals were assigned to Groups 1-4. Group 1 underwent euthanasia three days after stent placement. Group 2 underwent euthanasia seven days after stent placement. Groups 3 and 4 had their stents removed on day seven and were euthanized at day 14 and 21 respectively. The airways were evaluated grossly at each operative event for inflammatory changes, stent location, and occlusion and airway diameter. At necropsy, segments of stented and unstented trachea were sent for histopathologic and immunohistologic evaluation. Body weight was recorded at surgery, weekly and at necropsy. Tracheal diameter was assessed at insertion, removal and necropsy. Inflammatory response and location was noted grossly at endoscopy and necropsy and histologically.

The Palmaz Stent (Johnson & Johnson Interventional Systems Co, Warren, NJ) was selected for this study based upon its ease of use, use in pediatric patients and limited tissue reactivity.^{21,25} The Palmaz stent is a balloon expandable metallic stent composed of stainless steel filaments in a tubular mesh form. This design allows the diameter to expand while its length contracts. Stents utilized in this study were 30mm in length by 3.1mm in diameter when closed. Upon insertion and deployment with a balloon at 10ATM the stent expanded to 10mm in diameter and 20mm in length.

Stents were inserted through a 3.5 rigid bronchoscope (Karl Storz Endoscopy-America Inc.). After the animal was anesthetized, as described above, placed into a prone position, the vocal folds were visualized, and the animal was intubated with the bronchoscope. The bronchoscope was advanced under direct

visualization to the level of the carina; it was then withdrawn to approximately 2-3 cm above the take-off to the right upper lobe bronchus. Photographic documentation was obtained with a telescope (Hopkins II, Karl Storz Endoscopy-America Inc.). A Cordis PTA Balloon Catheter 10mm x 3cm (Cordis, Miami Lakes, FL) with stent was passed through the bronchoscope, along with a 0 degree 2.9mm telescope (Hopkins, Karl Storz Endoscopy-America Inc.), until it was located over the take-off for right upper lobe bronchus. The balloon was inflated to 10mm (ATM) pressure while being monitored manometrically. After deflation, the balloon was withdrawn from the bronchoscope. Photodocumentation of stent placement was obtained.

After intubation with the rigid bronchoscope a foreign body forceps (Alligator, Karl Storz Endoscopy-America Inc.) was inserted through the bronchoscope, telescope, to the level of the proximal edge of the stent. The stent placement, granulation tissue and mucosal reactivity were assessed prior to removal. While grasping the leading edge of the stent, the forceps were rotated clockwise until the stent collapsed upon itself. The stent was withdrawn through the bronchoscope and the placement site reassessed. Photodocumentation was obtained before and after extraction.

All animals underwent bronchoscopy with photodocumentation utilizing the same technique as previously described moments prior to being euthanized. Prior to being euthanized, animals were sedated with Torbugesic intramuscularly prior to receiving an intracardiac injection of potassium chloride. Each animal had their trachea and bronchi retrieved for evaluation. The specimens were transversely sectioned at the proximal end of the stent, intraluminal diameter were obtained at the proximal and distal segments along with the carina. 2mm sections were obtained and submitted for processing as hematoxylin and eosin stains (H+E) and immunohistological evaluation utilizing Vimentin, Fibronectin and Von Willebrand factor. (Sigma Chemical Co. St. Louis, Mo.). Inflammation was evaluated utilizing the scoring system derived by Wiswell and Wiswell³¹, along with a system created by the Veterinary Pathology Department at the Armed Forces Institute of Pathology.

Results:

There were no complications associated with the endoscopic procedure or stenting of the trachea. All experimental animals tolerated stenting without evidence of stridor or airway compromise. Stent integrity was maintained in all animals without occlusion, collapse or migration.

All study animals demonstrated appropriate weight gains. Utilizing a paired student t-test the tracheal diameter and growth were not affected by stent placement

Three days after stenting 14% (1/7) of group 1, animals had minimal (1/4) granulation tissue at the proximal end of the stented segment. At seven days following stenting 42% (3/7) of group 2, 57% (4/7) of group 3 and 86% (6/7) of group 4 had minimal (1/4) granulation tissue in the same area. All granulation tissue disappeared after stent removal in-groups 3 and 4. Control animals did not develop granulation tissue. The inflammatory response, as determined upon review of the H&E slides by three independent pathologists, confirmed the endoscopic and gross findings. Utilizing ANOVA and Fischer Test comparing Group 2 inflammatory results to that of combined Groups 3 and 4, a mean score of 23.857 was obtained for Group 2 and 12.929 for groups 3 and 4. A P value of .0007 was obtained for ANOVA and .0002 for Fischer Test. When evaluating for the presence of fibroblasts through the use of vimentin staining in Groups 1 through 4, we found that the proliferation of fibroblasts in the distal segment of stented tissue. These results were consistent with fibroblast proliferation at the submucosa layer. The vimentin staining was most prominent in deep submucosal layer in Groups 1 and 2. In Groups 3 and 4, the vimentin staining was most evident in the superficial layers. Fibronectin deposition was present in these layers at the same time intervals. Von Willenbrands factor was present in this area as well, but more prominent superficially in the later stages of healing. Histological review demonstrated resolution of inflammatory response after stent removal. There was no evidence of cartilage invasion. Re-epithelialization was noted in group 3 and 4 specimens. The right upper lobe bronchus was free of

obstruction and no gross or microscopic signs of pneumonia were present in this bronchus even though the inlet was stented.

Discussion:

This report demonstrates that expandable metallic stents are well tolerated in the pig trachea when used short term. We were unable to demonstrate any long lasting adverse effects on the trachea or the animal as a whole. The stent induced mucosal injury rapidly healed following stent removal and tracheal growth was unimpaired. Expandable metallic stents, specifically the Palmaz stent, have met with success in selected cases, within the pediatric population.^{17,21,25,26} However, obstructing granulation tissue has complicated long-term and distal placement of these stents.^{19,27} The placement of stents in the pig trachea has not been associated with an abundance of granulation tissue. This could be due to several factors; use of the stent in the normal airway, accurate stent placement and sizing as well as less intrinsic airway motion in the proximal trachea, as compared to the bronchus. Other animal studies have demonstrated similar findings in short term stent use.^{19,28,29} Despite these findings the impact of long-term stent placement on the pediatric airway is unclear.

Placement of expandable metallic stents in the airway is straightforward. They can be placed under direct vision or fluoroscopic guidance. In this study we were able to accurately determine the diameter of the pig trachea and use an angioplasty balloon of the same dimensions to place the stent. This sizing may have contributed to the minimal inflammation seen in the stented segment. In the narrowed airway accurate endoscopic sizing is more difficult, especially when there is preexisting mucosal and/or structural anomalies (i.e. complete tracheal ring, congenital heart disease). To our knowledge there is no instrumentation available to reliably determine the diameter of the distal airway. Refinements in this area may allow for appropriate stent size in the abnormal airway. Using stents made of inert substances with an open mesh framework allowed us to place the stent across secondary bronchial openings (i.e. right upper lobe), without causing bronchial obstruction. This has been demonstrated in other studies.²⁵ Stent removal was accomplished without complication in all study animals. However, the stents do not always collapse

completely with rotation. The distal end remained expanded in some of the animals. While this was not significant in the trachea it could pose a problem in removal of stents from the bronchus, as has been reported.²⁵ This study did not evaluate the potential problems associated with long-term stent removal.

Respiratory epithelial healing in the setting of stent placement has not been extensively investigated. This study evaluated what occurs with short-term stent placement and subsequent removal. With stent placement the mucosa appeared to lose all function and form granulation tissue. Other models of respiratory epithelial healing with minimal trauma have demonstrated rapid mucosal migration to close small mucosal defects.²⁴ This study did not demonstrate this process. This may be due to several factors. The stent may have not been in place long enough for mucosal healing to occur or the removal of the stent at necropsy that may have caused mucosal disruption. Clinically stents placed long-term are felt to become incorporated into the mucosa.^{21,30} Our study did not demonstrate stent incorporation, just an inflammatory response. Possibly this inflammation would resolve with a longer period of stenting and the stent would be covered with functional respiratory epithelium. Immunohistochemistry demonstrated wave of fibroblast activity over time deep in the submucosal layer to the superficial respiratory epithelium, while the stent was in place. This process was followed by wave of neovascularity in the same regions that resolved after stent removal. The pattern of wound healing with long-term stenting is unknown. Further study is needed to more fully understand this process.

PHASE 2: Wound Healing in the Airway with Penetrating Tracheal Injury Alone.

Introduction:

Penetrating injuries to the trachea are frequently associated with tracheal instability, requiring a tracheal intubation. Using expandable metallic stents the potential for stabilizing the tracheal wall along with simultaneous repair of the endotracheal wall, may obviate the need for endotracheal intubation in this setting. In children with tracheal anomalies, to include complete tracheal rings or long segment tracheal

stenosis, repair is frequently performed using augmentative procedures of the endotracheal wall with subsequent short term stenting. In performing this phase we desire to describe the mechanics of stent placement in this situation as well as the short term surgical results following stent placement. Due to postoperative infections encountered during trial ends of phase three, this phase was performed using a perichondrial coverage of the tracheal opening to cut down the number of infections as well as prevent mediastinum.

Materials and Methods:

The protocol was approved by the Institutional Review Board and Animal Care Committee at our institution. Twenty-four four week old pigs (*Sus scrofula*), with an average weight of 11.9kg underwent single stage laryngotracheoplasty and stenting as follows:

Animals were premedicated with Ketamine hydrochloride (20mg/kg) and rompun (2mg/kg) prior to receiving general endotracheal anesthesia (isoflurane and intravenous Propafol 4-8mg/kg/hr). All animals received Dexamethasone 4cc and Clindamycin 100mg prior to surgery.

Animals were prepped and draped in sterile surgical fashion. A perichondrial graft was harvested from the dorsum of the right auricle and soaked for 10 minutes in Clindomycin. Gloves were changed, and a SSLTP was performed. The thyroid cartilage, cricoid, and trachea were exposed in the midline. A vertical incision was made through tracheal rings four through six. A 12mm Palmaz balloon expandable metallic stent (Johnson and Johnson Interventional Systems Co., Warren, NJ) was mounted on a angioplasty balloon catheter (12mmX3cm Cordis PTA balloon catheter, Miami Lakes, FL), passed through the endotracheal tube, and visualized through the surgical defect. The endotracheal tube was then withdrawn to the level of the subglottis, and the stent inflated to 12mm under direct visualization. This created a visible airway opening of uniform size. The balloon was deflated and removed, and the ETT advanced through the stent.

A double layered perichondrial patch was sewn into the defect with 5-0 Prolene interrupted sutures to create an airtight seal. The wound was closed in layers, and a Penrose drain and pressure dressing placed. The animals were then extubated and recovered from anesthesia. Each animal received Clindamycin 100mg twice a day for seven days (unless euthanized according to protocol). Drains and dressings were removed on postoperative day 1.

Animals were divided into two groups: Group 1 – 21 day animals (4 animals), Group 2 – thirty day animals (four animals). Groups 1 and 2 underwent penetrating tracheal injuries with stenting and were sacrificed on postoperative day 21 and 30, respectively. The tracheal operative site was harvested and histologic sections taken from proximal, middle, and distal graft sites. Sections were graded using a scoring system based upon work by Wiswell and Wiswell ³⁶, assessing inflammatory change. A 5-point severity score (range 0-4) was assigned for each specimen and a total mean score calculated (Table 1).

Group 1 and 2 animals underwent endoscopy on postoperative day 7, with inhaled anesthesia (isoflurane). The larynx was exposed, and a 0° Hopkins II fiberoptic telescope (Karl Storz, Karl Storz Endo-America Inc.) used to visualize subglottis and graft site. The reconstructed/stented portion of the airway was examined for patency, stent integrity, granulation tissue formation, and the presence/absence of infection. Photodocumentation was obtained. The stent was then grasped proximally with endoscopic forceps (Karl Storz, Karl Storz Endo-America Inc.) and rotated, thereby collapsing the stents and facilitating removal. Care was taken to grasp the stent away from the operative site, to avoid dislocation of the graft. Cottonoids soaked in either MTC (.5mg/ml) or placebo (normal saline) were placed over the operative site under endoscopic visualization and held in place for two minutes. The application was performed a total of two times for each animal. Treatment vials were randomized by the Pharmacy Department and blinded to the primary investigators and pathologist for the duration of the study. Each group had two animals that received placebo and two that received MTC.

Results:

Of the eight animals in the study, seven survived the procedures without any difficulty. In both groups, the surviving animals had an average of 8.2 pounds of weight gain during the study. The inflammation score in their tracheas was that averaged 7 in the 21 day group, in the 30 day group the average inflammation score was 7.7. The internal diameter of the time of harvestation was the same in both groups it averaged 12.6mm. Histologically there was no significant difference between the MTC and the placebo treated groups in both the 21 and 30 day animals. This was due to trachea collapse observe in 5 out of the 8 animals that were euthanized. The trachea took on an oval shape at the operative site. The tracheal opening that was expanded at the time of the initial procedure was closed down completely over the perichondrial graft. H+E standing showed no evidence for new cartilage growth within the perichondrium. There was no granulation tissue at the operative site just an abundance of fibrous tissue. The operative site had mucosalized completely with respiratory epithelium.

Discussion:

The goal of the tracheal repair following traumatic injuries is to provide stable airway without evidence for stenosis or tracheal instability. In this small pilot study the anatomy of the pig trachea resulted in significant tracheal collapse following tracheal injury. The cartilage of the pig trachea is overlapping circular in nature not C shaped as in humans. We felt that the disruption of the circular cartilage C structure allowed for tracheal collapse. The short stenting period in this phase was successful in maintaining a normal airway diameter however once the stents were removed the trachea did collapse and the expanded trachea defect contracted so much that at the time of euthanasia there was no visible tracheal injury site or neocartilage formation.

The animals tolerated the procedure in all respects, gained weight normally and had minimal evidence for infection. The one animal that died, died because of large amount granulation tissue in the airway shortly after stenting. Microscopic evaluation of the tracheal specimens revealed no significant problems with

reepithilization or healing of the airway. However the samples size are so small that we could not tell the difference

between the MTC treated animals versus the Placebo. Additionally, the fact that the operative site contracted, we were unable to truly identify the impact of MTC on the fibroblast within this region.

In conclusion, this pilot study showed that stenting of the trachea in an acute setting can be performed without difficulty. Air leakage through a disrupted tracheal wall can be repaired safely with perichondtion in this setting. However the period of stenting in this pilot study was not long enough to prevent tracheal collapse. Further evaluation of the duration of stenting and the type of material used to close trachea defects must be performed to clearly understand the healing process occurring with tracheal injuries alone.

PHASE 3: Wound Healing in the Airway with Augmentative Reconstructive Procedures (i.e. auricular cartilage).

Introduction:

Management of subglottic stenosis in children remains a difficult problem. The goals of any treatment plan are maintenance of a patent airway, glottic competence, and voice quality. Several treatment options exist for laryngotracheal reconstruction (LTR)).³² To date, no one treatment option has been completely satisfactory. In recent years, single stage laryngotracheoplasty (SSLTP) has gained growing support, allowing for reconstruction of the stenotic airway and compression of the prolonged stenting period required with traditional LTR into a shorter period of endotracheal intubation. However, postoperative endotracheal stenting increases the risk of granulation tissue formation and restenosis. Additionally, maintaining the airway in these children during the stenting period can be difficult. Various adjunctive measures have been used to control and eliminate problems with wound healing. Most recently, the use of MTC, a potent fibroblast inhibitor, has been reported in conjunction with LTR.²

Recently, we demonstrated in an animal model pilot study that immediate extubation could be performed after SSLTP with the use of expandable metallic stents, thereby alleviating the need for prolonged endotracheal stenting and providing a realistic means of studying wound healing after SSLTP.³⁴ This study expands on those findings and seeks to determine the effects of mitomycin-C, a potent fibroblast inhibitor, on airway wound healing.

Materials and Methods:

The protocol was approved by the Institutional Review Board and Animal Care Committee at our institution. Twenty-four four week old pigs (*Sus scrofula*), with an average weight of 11.9kg underwent single stage laryngotracheoplasty and stenting as follows:

Animals were premedicated with Ketamine hydrochloride (20mg/kg) and rompun (2mg/kg) prior to receiving general endotracheal anesthesia (isoflurane and intravenous Propafol 4-8mg/kg/hr). All animals received Dexamethasone 4cc and Clindamycin 100mg prior to surgery.

Animals were prepped and draped in sterile surgical fashion. A wedge excision of auricular cartilage was performed, and the wound closed with 2-0 silk suture. The epithelium was removed from the graft, leaving the underlying auricular perichondrium intact. Using a template, a standardized augmentation graft (1.5x1.0cm) was fashioned and soaked in Clindamycin for 10 minutes. Gloves were changed, and a SSLTP was performed. The thyroid cartilage, cricoid, and trachea were exposed in the midline. A vertical incision was made through the cricoid cartilage and first two tracheal rings. A 12mm Palmaz balloon expandable metallic stent (Johnson and Johnson Interventional Systems Co., Warren, NJ) was mounted on a angioplasty balloon catheter (12mmX3cm Cordis PTA balloon catheter, Miami Lakes, FL), passed through the endotracheal tube, and visualized through the surgical defect. The endotracheal tube was then withdrawn to the level of the subglottis, and the stent inflated to 12mm under direct visualization. This created a visible airway opening of uniform size. The balloon was deflated and removed, and the ETT advanced through the stent.

The auricular cartilage graft was sewn into the defect with 5-0 Prolene interrupted sutures. The graft was placed to ensure that perichondrium lined the defect. Additionally, all sutures were placed extraluminally, as described by Cotton et al, to minimize the local inflammatory response.³² The wound was closed in layers, and a Penrose drain and pressure dressing placed. The animals were then extubated and recovered from anesthesia. Each animal received Clindamycin 100mg twice a day for seven days (unless euthanized according to protocol). Drains and dressings were removed on postoperative day 1.

Animals were divided into four groups: Group 1 – three day animals (three animals), Group 2 – seven day animals (three animals), Group 3 – fourteen day animals (six animals), and Group 4 – twenty-one day animals (six animals). Groups 1 and 2 underwent SSLTP with stenting and were sacrificed on postoperative day three and seven, respectively.

In all animals the laryngotracheal complex was harvested and histologic sections taken from proximal, middle, and distal graft sites. Sections were graded using a scoring system based upon work by Wiswell and Wiswell³⁶, assessing inflammatory change. A 5-point severity score (range 0-4) was assigned for each specimen and a total mean score calculated (**Table1**). Immunohistochemical staining was done for Vimentin, Fibronectine and Von Willebrand's factor.

Group 3 and 4 animals underwent SSLTP as previously described. On postoperative day 7, each animal underwent direct laryngoscopy and tracheoscopy with inhaled anesthesia (isoflurane). The larynx was exposed, and a 0° Hopkins II fiberoptic telescope (Karl Storz, Karl Storz Endo-America Inc.) used to visualize subglottis and graft site. The reconstructed/stented portion of the airway was examined for patency, stent integrity, granulation tissue formation, and the presence/absence of infection.

Photodocumentation was obtained. The stent was then grasped proximally with endoscopic forceps (Karl Storz, Karl Storz Endo-America Inc.) and rotated, thereby collapsing the stents and facilitating removal. Care was taken to grasp the stent away from the graft site, to avoid dislocation of the graft. Cottonoids

soaked in either MTC (.5mg/ml) or placebo (normal saline) were placed over the graft site under endoscopic visualization and held in place for two minutes. The application was performed a total of two times for each animal. Treatment vials were randomized by the Pharmacy Department and blinded to the primary investigators and pathologist for the duration of the study. Each group had three animals that received placebo and three that received MTC. Group 3 animals were sacrificed on postoperative day 14 and Group 4 animals on day 21.

An additional group of eight animals was prepared for scanning electron microscopy studies to document mucosal surface characteristics at the surgical site. Two additional animals for each experimental group were treated as previously described. The laryngotracheal complex was harvested in its entirety and placed in gluteraldehyde prior to sectioning.

The following surgical procedure was performed. Once the animals were anesthetized in sleep and prepped and draped sterilely an opening was made in the intratracheally between tracheal rings 4-7. A metallic stent was placed in this location expanding the trachea as described in expanding the trachea to 10 mm. Pericardium is harvested from the auricle of that pig and sewn in place to fill in the deep effect. The sutures were placed extra luminely and the seal was air tight at the end of the procedure. Pericardium was placed in a double layer over the tracheal defect. The eight animals were divided into two groups. The first group underwent the procedure followed by stent removal at 7 days. Two animals were treated with MTC and two with placebo topically at the time of stent removal. After 21 days these animals were euthanized. The second group underwent the same procedure except that they were euthanized at 30 days. All operated tracheal specimens were harvest at the time of euthanasia and underwent H+E staining with transverse sectioning. Measurements were taken on tracheal diameter at that time.

Results:

In general, animals demonstrated an acute inflammatory response to the stenting with the maximal amount of granulation tissue seen at the proximal graft site. This observation was noted both grossly and

endoscopically. No animal was found to have airway obstruction secondary to granulation tissue formation. There was a gradual resolution of the granulation tissue over time after the stents were removed, noted histologically. Additionally, the presence of skin infection/graft site infection also tended to resolve over time, regardless of the treatment group. Virtually all animals had some degree of a seroma or localized skin infection at 3-7 days postoperatively. By 21 days, this had resolved in all animals.

Grossly, poor mucociliary clearance was noted in all groups, based on the finding of mucosal stasis at the graft site, and was confirmed histologically by the paucity of ciliated respiratory epithelium. All animals tended to gain weight during the study, with no significant difference between groups. Two thirds of animals demonstrated some degree of stent collapse, either at necropsy or endoscopic evaluation. One stent was seen to collapse with vigorous coughing of the animal during endoscopic evaluation. Tonometric evaluation of the strength of the stents demonstrated a sharp rise in the amount of compression when between 400 and 600gms of force were applied longitudinally to the stent.

Three day animals demonstrated an exuberant acute inflammatory response at the graft site, characterized by infiltration of polymorphonuclear cells and early granulation tissue. The majority of granulation tissue was at the proximal aspect of the graft site. There was a variable amount of graft necrosis with infiltration of inflammatory cells into the graft itself. Additionally, the stent was seen to induce epithelial erosion and submucosal fibroproliferative response with intervening areas of granulation tissue between the stent interstices. There was increased staining for vimentin in these areas as well. Fibronectin and Von Willebrands Factor were faintly present at the operative site in the submucosal layer. Submucosal glandular hyperplasia was seen in all animals, except in the areas immediately beneath the stent mesh. No reepithelialization of the graft site was seen, although there was evidence of squamous metaplasia. SEM demonstrated the stent being coated with a fibrin network with associated clumps of platelets. (Fig. 1) Average preoperative weight for this group was 12.5 kg, with an average weight gain of .9 kg. Average inflammatory score for this group was 15.3 (maximum score 36). Average airway diameter in this group was 12.1mm at necropsy.

Seven-day animals had an average weight gain of 2.1 kg. One animal died of fulminate graft site infection with resultant airway collapse on postoperative day 6. Histological examination revealed persistence of the acute inflammatory response seen in the three-day animals. Granulation tissue formation was more pronounced, with progression of the submucosal fibroproliferative response. Focal squamous metaplasia and hyperplasia, with loss of cilia was again noted. No reepithelialization of the graft site was noted. Graft necrosis was more pronounced in the central area of the graft. SEM demonstrated the stent being coated with a fibrin network with associated clumps of platelets. Bacterial infiltration of the graft site was also noted. Submucosal necrosis and fibroproliferative response under the stent mesh was more pronounced than in three-day animals. There was increased staining intensity for vimentin, fibronectine and Von Willabrand's in this region as well. Average inflammatory score for this group was 13.5. Average airway diameter in this group was 12.1mm at necropsy.

Untreated animals in the fourteen-day group had an average weight gain of 1.6 kg. As a group, these animals demonstrated near total liquefactive necrosis of the grafts with variable amounts of graft displacement. There was an attempt at native cartilage ingrowth to repair the surgical defect, with resultant malformation of the cartilage ring. Progressive maturation of the inflammatory response was also seen, with mononuclear cells and mature granulation tissue, especially at the proximal aspects of the graft site. Even though the graft site was histologically unstable, there was reepithelialization over the graft site in all animals with squamous metaplasia. Average airway diameter in the untreated group was 12.2mm. Early submucosal scarring could be seen at previous stent areas. Average inflammation score for untreated animals was 13.4. In animals treated with MTC, the average inflammation score was 11.8. This group had evidence of graft incorporation with cartilage proliferation from the graft itself, with an average airway diameter of 12.5mm. Mature granulation tissue was noted, similar to the untreated group. Reepithelialization of the graft site was noted, but no cilia were seen. SEM showed surface characteristics of squamous epithelium with focal areas of ciliated cells (**Figure 2**). MTC treated animals had an average inflammation score of 11.8. Both groups demonstrated persistent scar tissue in areas that had been underneath the stent mesh with immature fibroblasts and loss of submucosal glands. There was no significant difference in immunohistochemical staining between the untreated and treated groups.

Average weight gain for the untreated animals in the twenty-one day group was 3.3kg. Grossly, there was no evidence of infection at the skin surface or graft site, nor any evidence of granulation tissue. Histological examination revealed focal mature granulation tissue at the proximal aspect of the graft, as well as surrounding graft remnants. The acute inflammatory response had graduated to a more chronic variety, with mononuclear cell infiltrates seen. Graft necrosis was more complete than in the fourteen-day animals. Reepithelialization had occurred over the graft site, with hyperplastic respiratory epithelium as well as squamous metaplasia seen. Average diameter of the untreated animals in this group was 13.4mm, and the average inflammation score was 7.7. Twenty-one day animals treated with MTC demonstrated vascular ingrowth into the graft with reepithelialization over the graft site. There was intense staining for fibronectin and von Willebrands factor in the same region. Vimentin staining was present throughout the operative site. Central graft necrosis was noted, with the lateral aspects of the graft demonstrating the proliferative capacity. One animal in the treated group had evidence of bacterial infection of the graft with persistent acute inflammatory response. SEM showed surface characteristics of squamous epithelium with focal areas of ciliated cells. Average airway diameter in these animals was 12.8mm, with an average inflammation score of 8.4. Average weight gain in these animals was 4.3kg. Mucosal ridges were evident grossly in both groups, and corresponded to mucosal areas in previous contact with the stent mesh. Histologically, these areas consistently demonstrated a submucosal fibroproliferative response and loss of normal submucosal glandular architecture.

Discussion:

The goal of SSLTP is to create a safe, stable airway while limiting the amount of postoperative stenting, and presumably limiting postoperative complications and rates of recurrence. We developed an animal model in which SSLTR can be performed with immediate postoperative extubation, while still stenting the operative site. In this manner we closely approximated the clinical scenario one would expect in children undergoing repair of subglottic stenosis. Previous studies have demonstrated the validity of the pig model in the evaluation of airway surgery. The use of metallic stents in the lower airway, and more recently the upper airway, has also been demonstrated in several reports.^{34,35} A pilot study at our institution has

demonstrated the ability to immediately decannulate or extubate following SSLTP and stenting in a pig model (4). We have expanded that study to include the use of a biological wound healing modifier (mitomycin-C) which has recently been used in airway surgery ³³.

The ability to minimize postoperative complications following SSLTP would be a welcome adjunct to this procedure. The need for stenting, as well as the optimal duration of stenting, are still being examined ³². The use of an endolaryngeal expandable metallic stent in this study obviated the need for endotracheal intubation. In doing so, the risk of developing supraglottic stenosis secondary to intubation trauma is minimized. Additionally, the need for neuromuscular blockade, positive pressure ventilation, and sedation to maintain stable endotracheal intubation is removed. Not only does this reduce the complications related to prolonged ICU stays with endotracheal intubation, but cost to the patient and hospital is reduced as well. Additionally, this stent could be placed distal to the true vocal cords, preserving normal glottic function. Through the use of an angioplasty balloon of known outer diameter, the stent can be selectively enlarged to create an expanded airway. This expansion was maintained in all study animals. Following the period of stenting endoscopic removal was possible in all cases with minimal local trauma. All these characteristics make this technique and type of stent interesting. However, although the Palmaz stents are made of tantalum, an inert metal, all animals in our study demonstrated a significant inflammatory response to surgery and stenting. This coincides with the findings of previous studies demonstrating the inflammatory response to the Palmaz stent. The inflammatory response was most prominent at the proximal graft site, presumable due to increased tissue mobility in the immediate subglottic area with resultant trauma secondary to minute movement of the stent. Recent studies have demonstrated the efficacy of coated stents in decreasing acute inflammation and granulation tissue, a consideration for future studies of stenting after SSLTP. ³²

The finding of a submucosal fibroproliferative response in both groups of animals, manifested grossly by mucosal ridges, raises some concerns. This occurred in areas previously in contact with the stent mesh, and was not affected by the application of MTC. It is unknown if this finding is unique to the Palmaz stent, or if it occurs with any stent placement. Obviously, any circumferential scarring can narrow the diameter of

an already compromised airway. Additionally, these areas were relatively devoid of the normal submucosal glands found in unstented areas. This could contribute to the poor mucociliary clearance noted during the study, as well as potentially affect long-term functioning. Further study on the long-term effects of these stents is warranted.

One interesting finding of this study was the relatively small amount of force necessary to collapse the stent. As shown in Figure 1, between 400 and 600 gms of pressure can reduce the lumen of the stent by 50%. Approximately two-thirds of the animals in this study had some amount of stent collapse, seen either at necropsy (Groups 1 and 2) or endoscopy. One stent was even seen to partially collapse after vigorous coughing by the animal during endoscopy. If stenting following SSLTP is to be a viable treatment choice, more studies need to be conducted to find a stent design with more structural integrity, to prevent unwanted collapse and restenosis or acute airway compromise.

Another aspect of successful SSTLR is reepithelialization of the graft site. Heatley et al. demonstrated good reepithelialization and cartilage survival following tracheoplasty and auricular cartilage reconstruction in a rabbit model. They hypothesized that the thin nature of the auricular cartilage allowed for more efficient nourishment by the surrounding tissues. They additionally found that the auricular cartilage graft reepithelialized to 100% coverage by postoperative day 14, with 100% graft coverage with epithelium.^{35...} We did not have the same success in our study, probably due to several factors. First, the ventral surface of the pig ear has one layer of densely adherent epithelium and perichondrium resulting in a moderate amount of trauma to the cartilage during graft harvest. Previous studies have demonstrated that surgical trauma is the greatest contributor to cartilage resorption in rabbit auricular cartilage laryngotracheal grafting.³⁵ Second, their study did not include stenting of the graft site, which increases the local inflammatory response and may prevent adequate neovascularization of the graft site. Third, local infection of the graft site was seen to a variable extent in many of the animals, despite antibiotic coverage. All animals were kept in communal pens, with four animals per pen. Despite daily cleaning, these conditions are less than favorable for uncomplicated wound healing and may have contributed to the high rate of graft resorption in our study. Finally, the total area of their graft site was much smaller than in our study (10mmx4mm vs.

15mmx10mm, respectively), most likely contributing to their high rate of reepithelialization. Our study animals did not show functional respiratory epithelium until the stent was removed, contradictory to previous studies that showed reepithelialization within the stented section of airway.

Restenosis at the surgical site is associated with both short and long-term failure following SSLTR. MTC is an antitumor antibiotic derived from the bacterium *Streptomyces caespitosus*. It has been shown to have a potent antiproliferative effect on cultured human fibroblasts at a concentration of .04mg/ml, with cytotoxic effects at 1mg/ml.³⁷ Previous studies have demonstrated that single 5-minute exposures can inhibit fibroblast proliferation for 36 days in tissue culture. However, these effects are felt to be localized to the area treated, as fibroblasts in surrounding tissue are not effected.³⁸ It is unclear, the degree of liquefactive necrosis of the graft was markedly decreased in MTC treated animals. More importantly, all animals treated with MTC demonstrated some amount of cartilage proliferation from the graft itself. This finding was not observed in the untreated animals; especially evidenced by the immunohistochemical stains. By 21 days, MTC treated animals demonstrated smaller overall airway diameters; however, this is felt to be due to better graft incorporation with improved airway stability. The effect of MTC on airway wound healing may be due more to its enhancement of graft viability by promoting neovascularization as compared to its antiproliferative effect. Further study is necessary to delineate this complex process.

Our histologic grading scale is a useful tool for evaluating wound healing in this animal model (Figure 1). This scale is based on an accepted scale developed by Wiswell and Wiswell³⁶ to evaluate inflammatory changes in the airway. It incorporates several markers of tissue trauma and quantifies them based on severity of the findings. This gives the clinician the ability not only to compare animals across groups, but also within treatment groups, looking for differences in outcomes. Furthermore, it allows outcomes from different studies to be compared using a given set of criteria. Although this does not entirely solve the problem of inter-observer differences, it does provide a basis for comparison that until this time has been lacking. A standard grading scale may help future clinicians to decide the best treatment from a number of possibilities. As new treatments are developed to combat postoperative inflammation, a standard grading scale would be helpful in evaluating its efficacy.

In the situation of penetrating tracheal injury with perichondrial patching the anatomy of the pig tracheal did not allow the diameter of the stented airway to be maintained with short term stenting alone. This was probably due to the nearly complete overlapping tracheal rings found in the pig. These differ from the c-shaped tracheal cartilage in humans. The pig tracheal rings had a tendency to collapse once the ring was cut. Perichondrial patching, while preventing air leakage into the mediastinum, did not provide enough rigidity to maintain airway diameter following stent removal. If the period of stenting was lengthened the airway diameter may have been maintained.

Conclusion:

The short-term use of metallic stents induced minimal tissue reactivity in the pig trachea, as compared to controls. Stent removal led to rapid resolution of this inflammation. Many unresolved issues remain regarding airway stents use for longer periods. Inflammation control is essential in the development of an ideal long-term airway stent.

1. The pig airway is an adequate model for pediatric laryngotracheal stenosis with reconstruction. The anatomy, airway size, and rapid growth of these animals simulate those conditions found in the pediatric population. Although infection and graft resorption were a problem in our study, better control of environmental factors may improve the clinical outcome.
2. Metallic balloon expandable stents were successfully used following SSLTR and allowed immediate postoperative extubation and a means of studying airway wound healing. The ability to selectively expand the stent to a known diameter allows predictable enlargement of the airway and an accurate means of determining the stability of the reconstructed airway following stent removal.
3. Before stenting is widely clinically applied, the optimal stent design needs to be developed. A stent design that provides for maximal strength with minimal tissue reactivity is needed. The stent should be associated with minimal local tissue trauma, while maintaining patency and providing support for the healing airway.

4. Stenting in this study was associated with the development of mucosal scarring following stent removal. The clinical importance of this scarring is not known.
5. MTC may improve graft incorporation following SSLTP. Animals treated with MTC demonstrated cartilage growth from the graft and enhanced neovascularization, a finding not associated with untreated animals. There did not appear to be any effect on the local inflammatory response nor the formation of "stent scars." However, since full scar maturation takes many months, the final effect of MTC is not known. Further long-term studies are needed to quantify the effect of MTC on airway wound healing and determine the proper dosage and timing of MTC application.

PHASE 4: Wound Healing in the Airway following Tracheal Sleeve Resection with Primary Anastomosis.

Due to the large number of postoperative infections and findings encountered in Phase 3, this phase was not performed. The investigators did not think that more information would be gained from this experiment.

CONCLUSIONS

Phase 1: We demonstrated that stent placement within the pig trachea is easily performed. No long-lasting adverse affects on the trachea in short term stenting were detected. Stent placement was associated with inflammatory process that was manifested by a submucosal proliferation of fibroblasts with subsequent angiogenesis. This process reversed once the stents were removed. There was no evidence that the stents caused delay in tracheal growth. However, the piglet grows exceedingly rapidly and a minimal amount of damage to the trachea might not impair tracheal growth. When the stents were in place there was no evidence for functional respiratory epithelium around the stents. With prolonged stenting, ciliated

respiratory epithelium is felt to completely coat the stent. Further study, would have to evaluate whether this occurs as well as the long term effect of stenting of the airway has on the submucosal layer and tracheal cartilage.

Phase 2: We found that a tracheal incision was followed by tracheal collapse and a severe narrowing of the trachea when the stent was removed. A perichondrial patch closed the defect well, but did not provide adequate support to prevent tracheal collapse. Additionally, this substance did not demonstrate neocartilage growth. We think collapse is due to the type of cartilaginous rings found in the pigs' trachea. From this pilot study, we do not feel that perichondrium is an adequate augmentation graft for tracheoplasty in a pig model. Additionally, while the stent was well tolerated in this situation once the stent was removed the trachea did not maintain its normal anatomic configuration. The duration of stenting necessary to maintain the structural integrity of the trachea in this setting needs to be determined. Wound healing was not well studied in this phase due to operative site contractor and the absence of neocartilage formation.

Phase 3: We demonstrated that single stage laryngotracheoplasty can safely be performed on pigs. Stenting process was easily performed in this setting and stent removal was not a problem in short term stenting following a laryngotracheoplasty. Auricular cartilage grafts were stable other than the fact that some became infected postoperatively. The use of MTC to prevent significant scarring in the operative site was evaluated thoroughly. It was found that MTC may improve graft incorporation following single stage laryngotracheoplasty due to an enhanced cartilaginous growth in the surrounding area as well as neovascularization around the graft site. We did not specifically demonstrate that fibroblasts were inhibited within the operative site in pigs treated with the MTC as compared to placebo. One potential reason for lack of more significant findings related to MTC and placebo usage was that we did not allow the pigs to live long enough to see a fully healed airway. It is the author's speculation that if the pigs were allowed to live forty days postoperatively the respiratory epithelium lining the operative sites that would have been normal and the underlying cartilaginous graft structure would have been better preserved in the MTC group. This supposition is supported by SEM findings seen at the operative site of these animals. Once the stent was removed there was rapid coverage of the graft site with squamous epithelium. It appeared that the final stages of healing and the studied group that the squamous epithelium was becoming ciliated in nature.

Further study should evaluate the long term effects of MTC in airway wound healing. This would incorporate the use of this substance following a traumatic injury in an animal model with subsequent analysis of the healing process for a longer period. Other investigations could evaluate changes in stent design and composition to determine if this would; prevent stent collapse, allow for less inflammation and enhanced airway healing.

Since we did not demonstrate inhibition of fibroblasts with MTC usage this may not occur in airway healing as has been demonstrated in ophthalmologic surgery. Future investigations in airway wound healing must be performed to understand this process and how MTC affects it.

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Table 1. Histopathological Scoring System

PARAMETER	SCORE				
	0	1	2	3	4
Intraepithelial mucin loss	absent	focal	diffuse	-----	-----
Submucosal hemorrhage	absent	focal	diffuse	-----	-----
Surface epithelial changes	absent	minimal	severe	-----	-----
Infiltrates	absent	mild lymphoplasmocytic	severe lymphoplasmocytic	mild PMNs	severe PMNs
Epithelial erosions	absent	1 area	2-3 areas	>3 or extensive	total
Granulation tissue	absent	focal	moderate	small polyps	total
Serrated epithelium	absent	mild	prominent	-----	-----
Glandular hyperplasia	absent	<50%	>50%	-----	-----

